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Article

MicroRNAs Mediate Dietary-Restriction-Induced Longevity through PHA-4/FOXA and SKN-1/Nrf Transcription Factors

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Summary

Background: Dietary restriction (DR) has been shown to prolong longevity across diverse taxa, yet the mechanistic relationship between DR and longevity remains unclear. MicroRNAs (miRNAs) control aging-related functions such as metabolism and lifespan through regulation of genes in insulin signaling, mitochondrial respiration, and protein homeostasis.
Results: We have conducted a network analysis of aging-associated miRNAs connected to transcription factors PHA-4/FOXA and SKN-1/Nrf, which are both necessary for DR-induced lifespan extension in *Caenorhabditis elegans*. Our network analysis has revealed extensive regulatory interactions between PHA-4, SKN-1, and miRNAs and points to two aging-associated miRNAs, miR-71 and miR-228, as key nodes of this network. We show that miR-71 and miR-228 are critical for the response to DR in *C. elegans*. DR induces the expression of miR-71 and miR-228, and the regulation of these miRNAs depends on PHA-4 and SKN-1. In turn, we show that PHA-4 and SKN-1 are negatively regulated by miR-228, whereas miR-71 represses PHA-4.
Conclusions: Based on our findings, we have discovered new links in an important pathway connecting DR to aging. By interacting with PHA-4 and SKN-1, miRNAs transduce the effect of dietary-restriction-mediated lifespan extension in *C. elegans*. Given the conservation of miRNAs, PHA-4, and SKN-1 across phylogeny, these interactions are likely to be conserved in more-complex species.

Introduction

Dietary restriction (DR) [1] is the most effective way to reduce the severity of age-related phenotypes and to extend lifespan in diverse organisms [2–5]. For example, adult *Caenorhabditis elegans* maintained on plates with lower amounts of bacteria have a 15%–30% increase in lifespan [6, 7], and *C. elegans* with *eat* mutations or subjected to dietary deprivation early in adulthood have lifespans lengthened by up to 50% [8–10]. Despite extensive study, however, the mechanisms behind DR-mediated longevity remain unclear.

The PHA-4 and SKN-1 transcription factors play key roles during DR and other stress-responsive processes to mediate longevity in *C. elegans* [11]. Specifically, PHA-4, a FOXA homolog necessary and sufficient for pharynx and foregut development, is required for DR-induced lifespan extension in *C. elegans* [11]. SKN-1 is a Nrf-related transcription factor required for endoderm formation in *C. elegans* embryogenesis and for the response to oxidative stress and starvation [12]. SKN-1 activates a wide range of genes involved in cellular repair, detoxification, and stress resistance [13]. SKN-1 expression in ASI sensory neurons has also been shown to be required for DR-induced lifespan extension [14]. SKN-1 and PHA-4 interact in a complex manner: for example, although DR does not further extend lifespan of *let-363/torc1* mutants, the autophagic response induced by TOR inhibition requires PHA-4 activity, and TORC1 and SKN-1 function in a feedback loop to activate stress resistance and other protective genes in longevity [15, 16]. In addition, SKN-1 can be directly inhibited by insulin/insulin-like signaling (IIS) pathway kinases in a similar manner as DAF-16/FOXO, and when translation is inhibited, SKN-1 and DAF-16 function together to promote stress resistance and longevity [17].

We suspected that the complex and context-sensitive interplay of these nutrient-responsive pathways is specifically orchestrated by a set of regulatory factors that integrate across many different pathways. MicroRNAs (miRNAs) represent a class of regulatory molecules that is well suited to linking multiple pathways in order to add robustness to gene expression networks [18–22]. miRNAs regulate many targets in a combinatorial fashion [23] because they target mRNAs through complementarity to a short seed sequence; conversely, individual mRNAs may be targeted by many miRNAs [24]. miRNA-targeted mRNAs are degraded or are translationally repressed [24, 25]. The expression of miRNAs is tightly regulated during development and in response to specific conditions, such as stress [26–28]. Thus, miRNAs have the capability of regulating many different pathways in a complex, subtle, combinatorial, and context-sensitive fashion, which are precisely the conditions observed at the intersection of nutrient sensing and aging biology.

Furthermore, miRNAs are important regulators of aging [22, 28], which also involves complex genetic pathway interactions. The role of miRNAs in these processes was established when it was shown that the *lin-4* miRNA regulates lifespan in *C. elegans* [29]. It was also shown that knockdown of *alg-1*, a *C. elegans* Argonaute gene, results in a significantly shorter lifespan compared to wild-type, indicating that miRNA maturation and function regulate longevity [26]. In fact, many miRNAs are significantly upregulated or downregulated with age [30]. Advances in high-throughput technologies such as deep sequencing have facilitated miRNA expression studies across time and in different conditions. Additional miRNAs that modulate aging were discovered via such strategies, including miR-71, miR-238, miR-239, and miR-246 [30].

Although these miRNAs do not affect developmental progression of *C. elegans* [30, 31], *mir-71*, *mir-238*, and *mir-246* loss-of-function mutants have a significantly shorter lifespan than wild-type, and overexpressing miR-71 or miR-246 increases lifespan; thus, these miRNAs promote longevity [30]. *mir-239* loss-of-function mutants exhibit an increase in

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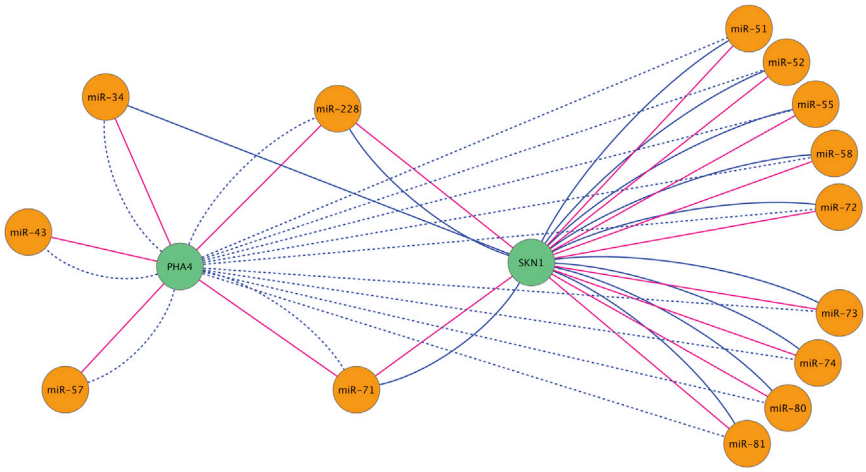

Figure 1. Aging Network of Transcription Factors and miRNAs

miRNA-mediated gene regulation, as predicted by mirWIP, is indicated by pink lines; blue lines indicate transcription factor-mediated gene regulation from modENCODE. Green nodes indicate transcription factors; orange nodes indicate miRNAs. Dotted line represents PHA-4-mediated regulation; solid line represents SKN-1-mediated regulation. Aging-associated miRNAs that comprise feedback loops with either PHA-4 or SKN-1 are shown. Only *mir-71* and *mir-228* both target and are targeted by PHA-4 and SKN-1. Network visualization was performed in Cytoscape. See also Figure S1 and Table S1.

lifespan compared with wild-type, and overexpressing miR-239 decreases lifespan, demonstrating that miR-239 antagonizes longevity [30]. Additionally, at least two of these miRNAs, miR-71 and miR-239, target components of the IIS and DNA damage checkpoint pathways to regulate aging. miR-71 targets the PI3K/AGE-1 and PDK-1 components of IIS, and miR-239 indirectly upregulates these genes [30]. Furthermore, the miRNA deletion mutants are either sensitive (*mir-71*, *mir-238*, and *mir-246*) or resistant (*mir-239*) to heat and oxidative stress in concordance with their respective lifespan phenotypes [30].

Further lines of evidence suggest that certain miRNAs coordinate responses to environmental perturbations and other stressors. Mutations to miRNAs in a sensitized genetic background cause embryonic and adult lethality [32], suggesting that many miRNAs may act to ensure developmental robustness [18–21]. In particular, miR-71 appears to integrate environmental inputs across many different possible life histories, acting to produce consistent developmental outcomes in each [33]. During *C. elegans* development, unfavorable conditions (e.g., food deprivation) cause differential expression of miR-71 and other miRNAs, including miR-34, miR-238, and *let-7* family members [33]. This suggests that these miRNAs might be necessary for the physiological response to stress or environmental conditions. Consistent with this, it has recently been shown that miR-71 is important for survival during and developmental recovery from starvation-induced growth arrest in the first larval stage [34]. In addition, genome-scale miRNA characterization efforts, which integrated miRNA target prediction with gene expression profiling of miRNAs and protein-coding genes during aging, revealed that many age-associated miRNAs appear to regulate aging-relevant processes of mitochondrial respiration and protein homeostasis [26, 35–38].

Results

A Network Analysis of Aging-Associated miRNAs

For complex, multipathway processes like aging, it is crucial to understand how the many constituent pathways interact to yield a particular phenotypic output. This task is often facilitated through the construction of (necessarily simplified) computational representations of the known networks of gene-regulatory interactions. Although aging networks focused on protein-protein interactions have been previously developed [39, 40], we constructed an aging network encompassing miRNAs and select transcription factors in a first attempt to capture the dynamic nature of changes that occur to regulatory factors during aging. We retrieved transcription factor binding data from the modENCODE project [41, 42] and determined targets based on the existence of at least one binding peak within 2 kb upstream and downstream of aging-associated miRNA gene loci. This criterion is less strict compared to that for a previously published regulatory network based on modENCODE data (1 kb upstream and 500 bp downstream of transcription start site [43]). Aging-associated miRNAs were determined by their common representation in two deep-sequencing expression-profiling studies [26, 30, 44]. We conducted target prediction using mirWIP [45], a relatively accurate and reliable target prediction algorithm that incorporates information regarding binding patterns of the RNAi-induced silencing complex in *C. elegans*.

Our initial framework for this network comprised the 21 transcription factors for which chromatin immunoprecipitation sequencing (ChIP-seq) data were available in modENCODE at the initiation of this project and 71 aging-associated miRNAs (Figure S1 available online). This small network identified novel aging candidate genes for further study, such as miRNAs that may comprise feedback loops and nodes that are particularly highly connected. Although the transcription factor ChIP-seq data were collected mainly during development, we believe that our network reports on aging rather than on development because of the inclusion of aging-associated miRNAs, many of which have aging-specific roles and no developmental roles [26, 30, 31]. Interestingly, *pha-4* and *skn-1* are the most highly connected nodes (Figure 1; Table S1). By examining miRNAs in the network, we find that miR-71 is among the most connected miRNA nodes, which agrees with experimental findings implicating miR-71 in many stress-responsive processes and lifespan regulation [34, 46, 47]. This concurrence suggests that other miRNAs identified based on network connectivity may similarly play important roles.

miRNAs Connected to PHA-4 and SKN-1

We used this regulatory-network approach to identify miRNAs with potential involvement in DR. Our network analysis reveals a set of miRNAs highly connected to *pha-4* or *skn-1*, transcription factors critical for the response to DR. Note that the ChIP-seq data from modENCODE were obtained from analysis of L1 *skn-1*::GFP animals and L1–L4 and young adult *pha-4*::GFP animals, which is why we confirmed these interactions in vivo

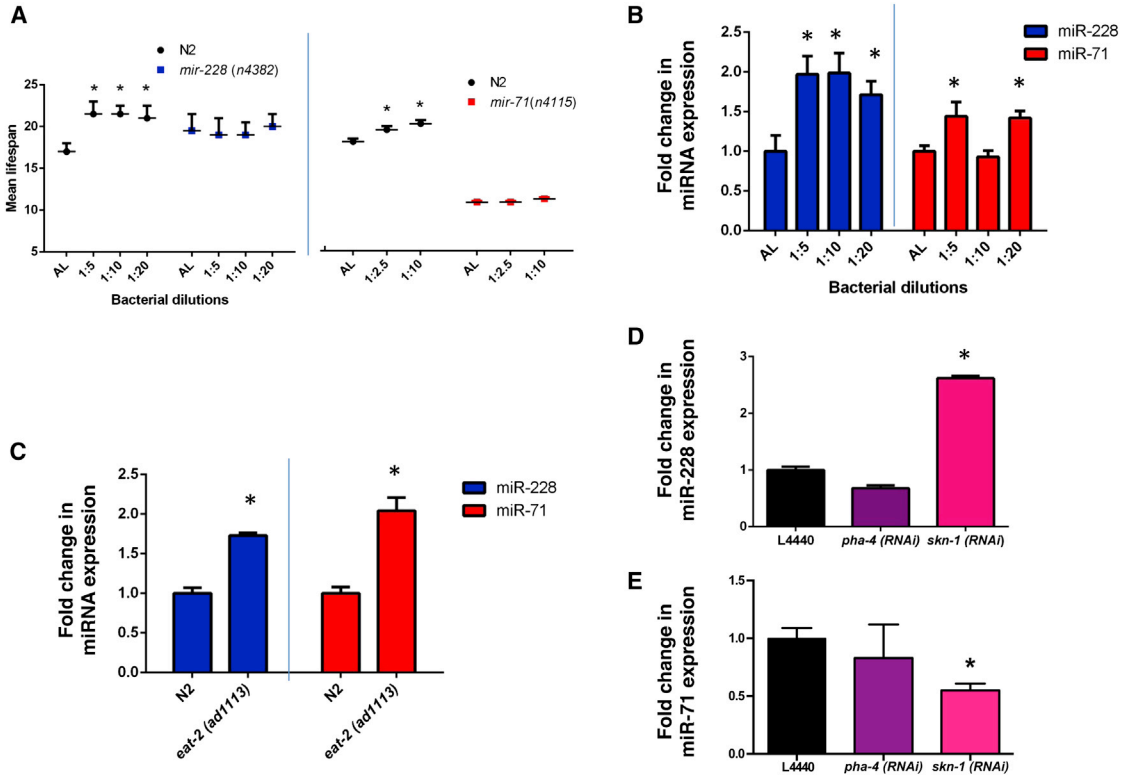

Figure 2. miRNAs Function during Dietary Restriction of Adult Animals

(A) miR-228 and miR-71 are required for dietary restriction (DR). *E. coli* (OP50) food was diluted 1:2.5, 1:5, 1:10, and 1:20 compared to the ad libitum (AL) concentration. Wild-type N2 animals fed diluted bacteria live significantly longer than N2 animals fed under ad libitum (*$p < 0.01$), as previously reported. By contrast, loss-of-function miRNA mutants *mir-228(n4382)* (left) and *mir-71(n4115)* (right) fail to exhibit this mean lifespan extension when fed diluted OP50, showing that miR-228 and miR-71 are required for DR response (see also Figures S2A and S2B).

(B) miRNAs are upregulated during DR compared to ad libitum. Mature miRNA expression in day 3 adult animals was measured by qRT-PCR (normalized to U18). Left: when N2 animals are fed diluted OP50, expression of miR-228 is increased compared to miR-228 levels under ad libitum. Thus, miR-228 may be activated in response to DR. This was also confirmed by GFP analysis (see Figure S2D). *$p < 0.05$, compared to AL. Right: miR-71 is similarly upregulated by DR induced by bacterial dilution. *$p < 0.05$, compared to AL.

(C) miRNAs are upregulated under DR in the *eat-2* mutant compared to wild-type as measured on day 3 of adulthood by qRT-PCR (normalized to U18). Similar to growing N2 animals on diluted OP50, mature levels of miR-228 and miR-71 are slightly higher in *eat-2(ad1113)* mutants, a genetic model of DR, as compared to a wild-type background. This alternative model of DR further indicates that the miRNAs are activated under DR conditions, as measured in day 3 adult animals via qRT-PCR. *$p < 0.05$, compared to N2 when measuring miR-71 and miR-228 levels.

(D) Opposite regulation of miR-228 mature expression by *pha-4* and *skn-1* in adult animals, as measured on day 3 of adulthood by qRT-PCR (normalized to U18). miR-228 expression (measured by qRT-PCR) is also affected by *pha-4* and *skn-1*, in which *pha-4* promotes expression of miR-228, whereas *skn-1* inhibits its expression, as compared to an empty vector RNAi control (L4440). Analysis of *mir-228*::GFP animals grown on *pha-4* and *skn-1(RNAi)* indicates that the PHA-4 and SKN-1 transcription factors may be directly targeting the *mir-228* gene (see Figure S2F). *$p < 0.05$, compared to L4440.

(E) *skn-1* is required for maximal expression of miR-71 in adult animals. In a *skn-1(RNAi)* background, miR-71 is downregulated, as compared to an empty vector RNAi control (L4440) at day 6 of adulthood (relative to RNA6B). *$p < 0.05$, compared to L4440.

Error bars show SEM.

in aging animals [41, 42]. These miRNAs not only appear to be downstream targets of these transcription factors, but also are predicted to feed back to directly regulate the expression of these factors (Figure 1). Specifically, our network analysis shows five age-associated miRNAs predicted to target *pha-4* and 11 miRNAs predicted to target *skn-1*. The modENCODE ChIP-seq data set reveals 45 age-associated miRNA genes bound by PHA-4 and 25 miRNA genes bound by SKN-1, with 24 miRNAs in common. As shown in Figure 1, miR-71 and miR-228 are the only miRNAs that are targeted by and are themselves predicted to target *pha-4* and *skn-1*. This suggests that these two miRNAs and these two transcription factors function in regulatory feedback loops.

The central location of miR-71 and miR-228 in our network and their association with PHA-4 and SKN-1, which are required for the response to DR [48], led us to test whether these miRNAs might have functional roles during DR. We obtained knockout mutants for the *mir-71* and *mir-228* miRNA genes and conducted lifespan assays under ad libitum (AL) feeding and across a range of conditions that produce DR. If the miRNA genes were important for lifespan extension due to DR, then we would expect DR to have no effect on the lifespan of the miRNA mutants. As expected, wild-type *C. elegans* exhibits a significantly longer lifespan across various conditions of DR (Figure 2A). As previously observed, loss-of-function *mir-71(n4115)* mutants have a significantly shorter lifespan than wild-type animals [30, 46]. However, in contrast to wild-type animals, the lifespan of *mir-71* mutants is not extended by DR, showing that miR-71 is required for the DR response (Figures 2A and S2B). We also tested the function of *mir-71* using *eat-2(ad1116)*, a genetic model of DR, and we observed that the absence of *mir-71* suppresses the

longevity of *eat-2(ad1116)*, which again demonstrates that miR-71 is required for DR-induced longevity (Figure S2C). Similarly, *mir-228(n4382)* mutants exhibit an abnormal response to DR. Whereas loss-of-function *mir-228* mutants have a significantly longer lifespan than wild-type animals (demonstrating that miR-228 normally antagonizes lifespan; see also Figure 4A), the lifespan of these *mir-228* mutants is not further extended by DR (Figures 2A and S2A). Suppression of the DR response in *mir-71* and *mir-228* mutants suggests that both of these miRNAs are required for the lifespan-extending effects of DR.

In order to understand the molecular mechanism underlying the functional roles of miR-71 and miR-228 in DR, we examined whether the expression of these miRNAs is altered during conditions of DR. We found that the expression of miR-71 and miR-228, as measured by quantitative RT-PCR (qRT-PCR) and promoter::GFP reporter expression, is upregulated when adult animals are reared in nutritional conditions that are sufficient to elicit DR-mediated extension of lifespan (Figures 2B and S2D). In addition, an *eat-2(ad1113)* genetic model, which mimics DR, similarly leads to upregulation of the levels of miR-71 and miR-228 (Figure 2C). By conducting pharyngeal pumping assays of *mir-71* and *mir-228* animals using N2 and *eat-2* as controls, we confirmed that *eat-2* animals pump significantly slower than N2, whereas N2 animals pump slower as they approach mid-adulthood (Figure S2E). Importantly, *mir-71* and *mir-228* animals pump normally like N2 animals, except this rate declines when many of the animals are near death (i.e., *mir-71* animals around day 10) (Figure S2E). These pumping rates during aging confirm the normal pumping rates observed for *mir-71* and *mir-228* animals during development [31]. Moreover, it is evident that the observed effect of miRNAs regulating DR-mediated longevity is due to a mechanism independent of pumping rate.

Because *mir-71* and *mir-228* are likely transcriptional targets of SKN-1 and PHA-4 (Figure 1), we considered whether regulation of these miRNAs might depend on these transcription factors. Indeed, RNAi directed against *skn-1* leads to an alteration in mature levels of both miR-228 and miR-71, and *pha-4(RNAi)* causes a reduction of the expression of mature miR-228 but does not affect expression of miR-71 (Figures 2D and 2E). *pha-4(RNAi)* also causes a decrease in *mir-228*::GFP expression, whereas *skn-1(RNAi)* leads to an increase in *mir-228*::GFP expression (Figure S2F). These results show that SKN-1 and PHA-4 are required for the expression of miR-71 and miR-228, respectively, whereas SKN-1 represses miR-228. The opposite effect of SKN-1 on the expression of miR-228 and miR-71 is consistent with the opposing roles of these miRNA genes on lifespan (Figure 2A).

Because our network analysis suggested possible bidirectional regulation between these miRNAs and PHA-4 or SKN-1, we looked for genetic interactions between these factors by examining the effects of knocking down *skn-1* or *pha-4* on the phenotypes of these miRNA mutants. We observed that the long lifespan of *mir-228* mutants depends on both *pha-4* and *skn-1* (Figure 3A), suggesting that, normally, miR-228 antagonizes lifespan by inhibiting PHA-4 and SKN-1. Indeed, the loss of *mir-228* actually shortens the lifespan of animals when *pha-4* or *skn-1* is knocked down. Similarly, we tested the interaction of miR-71 with SKN-1. Because both *skn-1* and *mir-71* mutants have a short lifespan, we tested a miR-71 overexpressing array [30] for genetic interactions with *skn-1*. We found that miR-71 overexpression suppresses the *skn-1(RNAi)* phenotype (Figure 3B). Although animals reared on *skn-1(RNAi)* are usually shorter lived compared to wild-type, in the context of miR-71 overexpression, they actually live longer (Figure 3B). This result indicates that the function of SKN-1 depends on miR-71 and is consistent with our observation that SKN-1 is required for proper miR-71 expression (Figure 2E).

To test whether miR-228 negatively regulates PHA-4 or SKN-1, we examined the expression of these factors in *mir-228* mutants. We observed that *pha-4* and *skn-1* mRNA levels are upregulated in *mir-228* mutant animals under both DR and ad libitum conditions (Figures 3C and S3A). Because miR-228 levels are altered by RNAi against *pha-4* and *skn-1* (Figure 2D), this indicates a possible feedback loop. Consistent with this model, *pha-4*::GFP expression and *skn-1*::GFP expression are also upregulated in a *mir-228* background (Figure 3D), which was observed under both DR and ad libitum conditions (Figure S3B). These data strongly implicate miR-228 as a mediator of lifespan and, in particular, show that miR-228 negatively regulates *pha-4* and *skn-1*. We also tested whether miR-71 regulates *pha-4* and *skn-1* using molecular and genetic approaches. We examined the expression levels of *pha-4* and *skn-1* and observed that only *pha-4* levels were increased in *mir-71* mutants, as measured via qRT-PCR (Figure 3E) and GFP fusion (Figures 3F and S3C–S3H), suggesting that miR-71 targets and negatively regulates *pha-4*.

miR-228 Is Required for Normal Aging and Heat Stress Response

Because both miR-71 and miR-228 are required for DR-mediated longevity, we asked whether these miRNAs may also be required for regulating lifespan under ad libitum conditions as well. Although miR-71 had been previously identified as promoting longevity and stress resistance [30], the function of miR-228 was unknown. In contrast to *mir-71* mutants, we observed that *mir-228* mutants exhibit an extended lifespan (Figure 4A) and are resistant to heat stress (Figure 4B). Conversely, three separate lines of *mir-228* overexpressors (coinjected with *myo-3*::GFP) displayed the opposite phenotypes from *mir-228* mutants, in which these overexpressors were short lived (Figure 4A) and were more sensitive to heat stress (Figure 4B) than N2 or *myo-3*::GFP animals were. We confirmed overexpression of mature miR-228 in these three lines using TaqMan assays (Figure S4A).

These results suggest that miR-228 is normally required to antagonize longevity and stress resistance. *mir-228* mutants were previously shown to not be sickly or exhibit developmental or viability abnormalities [31]. We confirmed that these mutants are developmentally normal by observing the same timing of progression through larval stages for mutant and wild-type animals, as well as by observing a normal brood count (Figure S4B). We also saw that *mir-228* mutants exhibit faster body bend movements and a slower increase in gut autofluorescence compared to wild-type animals (Figures S4C and S4D), indicating that, in fact, these long-lived animals also age more slowly. We speculate that the long-lived phenotype of *mir-228* mutants is therefore due to a slower rate of aging in these adult animals.

Because a number of miRNAs that regulate lifespan also exhibit dynamic expression changes during aging [30, 31], we investigated whether miR-228 was upregulated or downregulated in aging animals compared to young adult animals. We examined a *mir-228*::promoter::GFP fusion [50] to monitor expression of this miRNA in adult animals. We observed that *mir-228*::GFP expression increases during early adulthood, reaches a peak, and then begins to slowly decrease in mid

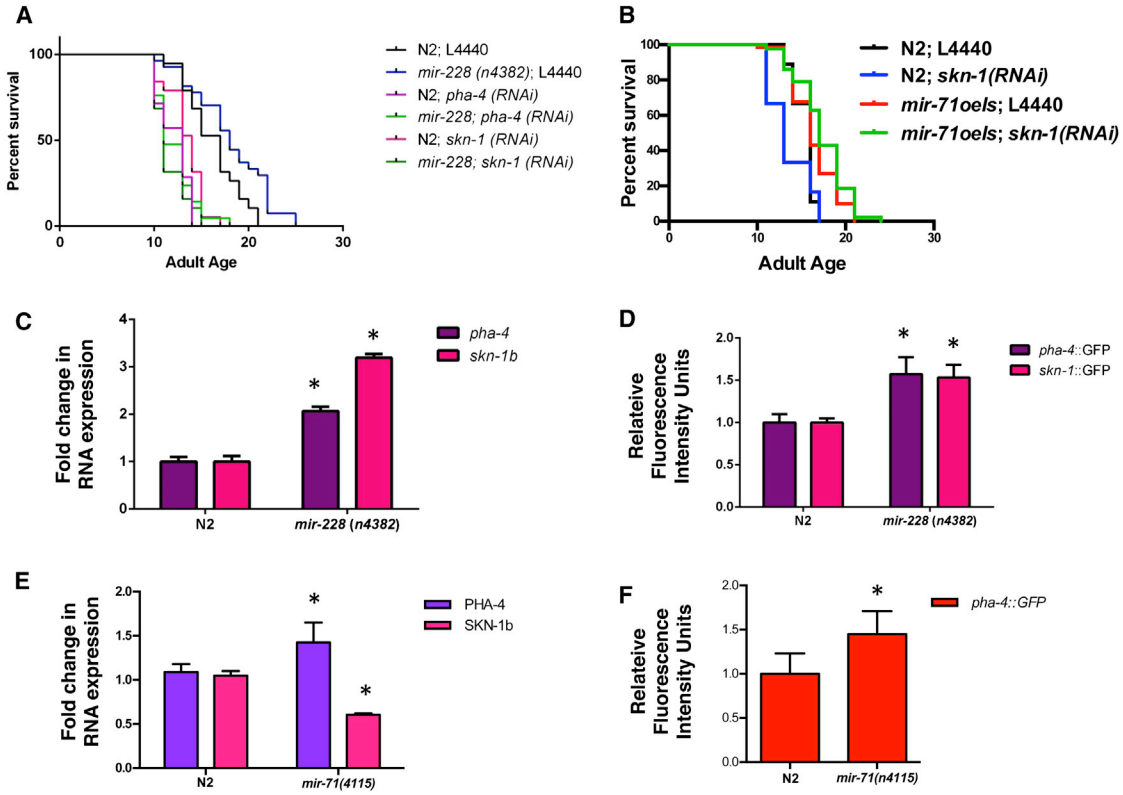

Figure 3. miRNAs Interact with PHA-4 and SKN-1 to Affect Longevity

(A) Whereas *mir-228* mutants grown on empty vector RNAi (L4440) are long lived, *mir-228* animals fed *pha-4(RNAi)* or *skn-1(RNAi)* during adulthood are short lived ($p < 0.05$), similar to *pha-4* and *skn-1(RNAi)* alone. Therefore, *mir-228* requires wild-type *pha-4* and *skn-1* to affect longevity and may act upstream of these transcription factors.

(B) *mir-71* overexpression, which causes animals to be long lived, suppresses the short lifespan induced by *skn-1(RNAi)* ($p < 0.05$). Thus, *mir-71* may function downstream of *skn-1*. oels, overexpression integrated strain.

(C–F) *pha-4* and *skn-1* mRNAs are targeted by miR-71 and miR-228. Error bars show SEM.

(C) *pha-4* and *skn-1* levels are increased in *mir-228* mutant adults as measured by qRT-PCR, indicating that they may be targeted by this miRNA. This was true under both ad libitum and DR conditions (see Figure S3A). qRT-PCR was performed on day 3 adults and normalized to the geometric mean of *cdc-42*, *pmp-3*, and *Y45F10*. *$p < 0.05$, compared to N2.

(D) Using transgenic lines containing the endogenous 3′ UTRs of *pha-4* and *skn-1*, respectively, analysis of *pha-4*::GFP and *skn-1*::GFP expression validates that levels of these transcription factors are increased in *mir-228* mutants. This held true under both ad libitum and DR conditions (see Figure S3B). Fluorescence was compared between *pha-4*::GFP or *skn-1*::GFP in *mir-228* mutant relative to N2 day 3 adults. *$p < 0.05$, compared to N2.

(E) *pha-4* levels are increased in *mir-71* mutant adults as measured by qRT-PCR, indicating that *pha-4* may be targeted by this miRNA. *skn-1* levels are slightly downregulated in *mir-71* adults compared to N2 adults, perhaps through an indirect targeting mechanism. qRT-PCR was performed on day 3 adults and normalized to the geometric mean of *cdc-42*, *pmp-3*, and *Y45F10*. *$p < 0.05$, compared to N2.

(F) Analysis of *pha-4*::GFP expression validates that levels of this transcription factor are increased in *mir-71* mutants (see also Figures S3C–S3H). Fluorescence was compared between *pha-4*:: in *mir-71 mutant* versus N2 day 2 adults. *$p < 0.05$, compared to N2.

to late adulthood (Figure 4C). Interestingly, in a DNA microarray study, the changes in PHA-4 expression during adulthood exhibited a similar pattern, supporting our findings that changes in PHA-4 expression during aging may directly alter patterns of *mir-228* transcription during aging as well [49]. To confirm these results and to look more closely at the expression patterns of these factors, we used qRT-PCR to measure the expression of PHA-4, SKN-1, miR-71, and miR-228 during adulthood (Figure 4D). We found that levels of PHA-4 and miR-71 exhibit a dramatic increase in the first 5–6 days of adulthood, confirming previous reports [30, 49]. Mature miR-228 levels exhibit the same dynamic expression pattern as seen via *mir-228*::GFP expression, in which there is a strong upregulation of miR-228 in early adulthood, followed by a decrease in expression in mid to late adulthood (Figure 4D). The above data indicate that, like miR-71, miR-228 coordinately regulates DR, longevity, and stress resistance.

Discussion

DR has previously been shown to extend the lifespan of several species, and it can delay the onset of aging-associated diseases. However, little is known regarding factors that are required for DR-mediated longevity. In a novel mechanism, we show that two *C. elegans* miRNAs, miR-71 and miR-228, are required for this effect. These miRNAs are upregulated under DR, and through their interactions with PHA-4/FOXA and SKN-1/Nrf transcription factors, miR-71 and miR-228 regulate the long-lived phenotype of dietary-restricted animals. Based on our findings, we have discovered new links in the important pathway connecting DR to aging, in which miR-71 and miR-228 repress the expression of PHA-4 and SKN-1, which are required for normal lifespan and DR effects (Figure 5).

By interacting with PHA-4 and SKN-1, miR-71 and miR-228 set in motion a genetic cascade to affect lifespan. We have

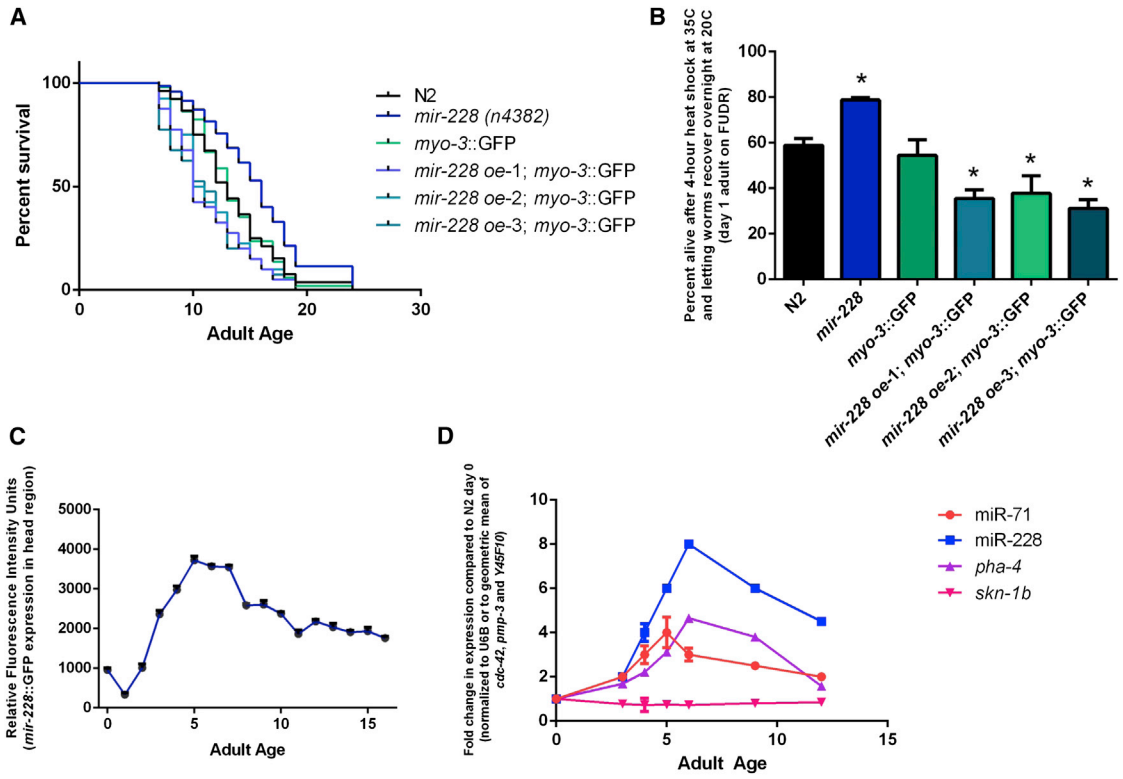

Figure 4. miR-228 Affects Longevity and Stress Response in *C. elegans*

(A) miR-228 antagonizes longevity in *C. elegans* because *mir-228* mutants are long lived compared to N2 wild-type ($p < 0.01$). Three lines of *mir-228* overexpressors coinjected with *myo-3*::GFP are shorter lived than N2 or *myo-3*::GFP ($p < 0.05$).

(B) *mir-228* mutants are resistant to heat stress. When day 1 adult animals grown on solid media are subjected to a 4 hr heat shock at 35°C, the percentage of *mir-228* animals surviving this stress is significantly higher than N2 (*$p < 0.05$). Conversely, the percentage of *mir-228* overexpressor animals surviving this stress is significantly lower than N2 or *myo-3*::GFP (*$p < 0.05$). This indicates that the longevity and stress response phenotypes of this miRNA are highly correlated. Error bars show SEM.

(C and D) miR-228 is dynamically expressed during aging. *mir-228*::GFP expression during aging (head region) is shown (C). GFP expression was quantified as a readout of *mir-228* levels, which peak in mid-adulthood. This same pattern holds true when quantifying *mir-228* in the head region (amphid and excretory cells), where expression is brightest, or quantifying expression across the entire animal. Expression of mature miR-71, miR-228, *pha-4*, and *skn-1* in wild-type animals over multiple aging time points via qRT-PCR is shown (D). *pha-4* follows the same pattern as shown by Lund et al. [49], in which levels peak at about day 6 adulthood before decreasing; miR-228 exhibits the same expression profile, indicating that PHA-4 promoting expression of *mir-228* is the stronger side of the feedback loop. As miR-71 levels are increasing, *pha-4* levels are lower than miR-71, but when miR-71 levels decrease, *pha-4* levels are higher, consistent with the model that miR-71 targets *pha-4*. Finally, *skn-1* levels remain constant during all aging time points, indicating that the opposing interactions of miR-228 targeting and downregulating *skn-1* while SKN-1 targets *mir-228* may effectively cancel each other out so that *skn-1* levels do not change. Error bars show SEM.

See also Figure S4.

shown that PHA-4 and SKN-1 promote *mir-228* and *mir-71* expression, respectively, whereas SKN-1 antagonizes *mir-228*. Conversely, miR-228 reduces both *pha-4* and *skn-1* mRNA levels, whereas miR-71 targets *pha-4* mRNA (Figure 5). We have shown previously [30] and in this work that miR-71 and miR-228 affect stress response in *C. elegans*. Because DR is a classic example of a specific stress that alters organismal lifespan, it would follow that these miRNAs should also be required for DR-mediated effects. These miRNA-transcription factor feedback loops are present under both ad libitum and DR conditions, resulting in an overall increase in lifespan that is most likely due to the additional effects of other factors yet to be determined.

Although this model shows multiple feedback interactions between expression of miRNAs and transcription factors based on the above experiments, it does not address the question of how these interactions result in the overall expression patterns of these miRNAs and transcription factors in wild-type conditions. We observed changes in expression of mature miR-71, miR-228, *pha-4*, and *skn-1* in wild-type animals over multiple aging time points via qRT-PCR. *pha-4* follows the same pattern as shown by Lund et al. [49], in which levels peak at about day 6 adulthood before decreasing; miR-228 exhibits the same expression profile (Figure 4D), indicating that PHA-4 promoting expression of *mir-228* is the stronger side of the feedback loop. Additionally, miR-71 peaks at about day 5 in adulthood; however, the result is that as miR-71 levels are increasing, *pha-4* levels are lower than miR-71, but when miR-71 levels decrease, *pha-4* levels are higher (Figure 4D). Thus, the result reflects miR-71 targeting *pha-4*. Moreover, *skn-1* levels remain constant (close to its levels on day 0 adulthood) during all aging time points and do not significantly increase, indicating that the opposing interactions of miR-228 targeting and downregulating *skn-1* while SKN-1 targets *mir-228* effectively cancel each other out so that *skn-1* levels do not change (Figure 4D). However, SKN-1 regulating

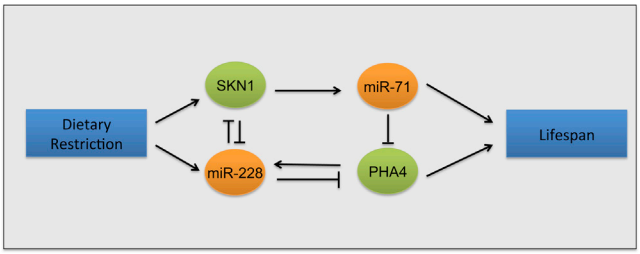

Figure 5. A Model for How miR-71 and miR-228 Interact with PHA-4 and SKN-1 to Mediate the Effects of DR, Leading to Increased Lifespan

DR promotes the expression of miR-71 and miR-228, which then function in feedback loops with PHA-4 and SKN-1 simultaneously. miR-228 targets both *pha-4* and *skn-1*, and PHA-4 promotes *mir-228* expression, whereas SKN-1 antagonizes *mir-228* expression. Conversely, SKN-1 promotes *mir-71* expression, and miR-71 targets *pha-4*. These feedback loops fine-tune the DR response in *C. elegans* to promote lifespan.

mir-71 may not be relevant in wild-type animals because its expression is constant while *mir-71* expression rises and falls, perhaps due to another mechanism.

Recent work has also shown an interaction between a *C. elegans* miRNA, miR-80, and DR, in which miR-80 appears to ensure that DR programs are not exhausted under ad libitum conditions [51]. In contrast to our findings with miR-71 and miR-228, however, miR-80 does not seem to be required for DR to extend lifespan [51]. Possibly independent of PHA-4 or SKN-1, miR-80 levels are decreased upon DR to promote activation of metabolic pathways [51]. Additionally, downregulation of Dicer and miRNA processing has also been observed with age in *C. elegans* and in mice, and these effects are prevented in both species by placing the animals under DR, indicating the possibility that additional miRNAs could be induced upon DR [52].

Although a few studies have attributed aging-associated and other functions to miR-71 in *C. elegans* [30, 34], this is the first report identifying a role for miR-228. In fact, miR-228 is highly conserved, and its mammalian homologs have been identified as the miR-96/182/183 family [53], which is critical for ciliated neurosensory organ development [54, 55]. Our findings demonstrate a role for miRNAs in DR of *C. elegans*, but given the conservation of miRNAs and PHA-4 and SKN-1 across species, these interactions may also be conserved in higher species. A few studies have reported numerous changes in the mammalian miRNA expression profile under DR [56–58]. Thus, it may also hold true that the miRNAs induced upon DR in mammals may be responsible for transducing the effects of lifespan extension in higher species as well. Because miRNAs are emerging as therapeutics in diseases of aging, this also brings up the possibility of using miRNAs to intervene in end-of-life care.

Experimental Procedures

Identification of Aging-Associated miRNAs

Lists of aging-associated miRNAs from the de Lencastre et al. [30] and Kato et al. [26] studies were combined, and only those miRNAs with greater than 20 sequence reads combined between day 0 and day 10 or 12, respectively, after read count normalization were considered for further analysis.

modENCODE TF-ChIP Data Analysis

Transcription factor-mediated gene regulation was determined by the presence of at least one transcription factor binding site within 2 kb upstream or downstream of the miRNA gene locus based on miRBase version 18 (http://www.mirbase.org) [59, 60].

DR Assays

Briefly, beginning with an OD_{600} of 0.5, OP50 *E. coli* was diluted 1:2.5, 1:5, 1:10, and 1:20 using M9 buffer to prevent additional bacterial growth. L1-starved synchronized mutant and N2 animals were grown to young adulthood on standard nematode growth medium plates and then transferred as young adults to ad libitum and diluted OP50 plates. Lifespan was assessed every 1–2 days.

qRT-PCR Analysis

miRNA expression levels were determined by qRT-PCR using TaqMan MicroRNA Assays (Applied Biosystems). The expression of *pha-4* and *skn-1b* mRNA was assayed using SYBR Green I according to manufacturer protocols (Roche). Primers were specifically designed for the *skn-1b* isoform because this isoform has been shown to be required for DR-mediated longevity [14].

GFP Analysis

Quantitative analysis of GFP fluorescence expression was performed on a Zeiss AxioPlan upright microscope. Outlines were drawn around fluorescent images of each animal using ImageJ. Whole-animal images were taken using the 10× objective, and head-only images were taken using the 40× objective. The mean and maximum GFP image intensity were calculated over the outlined animal area, and both measurements provided similar results.

Pharyngeal Pumping Assays

Pumping rate was defined as the number of contractions of the terminal bulb over 1 min. Animals were synchronized by bleaching. On each day of adulthood, the number of pumps of the terminal pharyngeal bulb was observed using a dissection scope, and ten different animals were chosen per strain each day (grown on 5-fluorodeoxyuridine plates). Only animals residing on the bacterial lawn were followed because pumping rates vary on and off the lawn.

Heat Stress Assays

Mutant and N2 animals were simultaneously exposed to a 4 hr heat shock in a 35°C incubator, and animals were allowed to recover overnight by transferring plates to 20°C. The number of surviving animals on each plate was then recorded.

Brood Size Assay

Fertility was measured by transferring five N2 and five *mir-228* animals post-L4 molt (~56 hr) to individual plates every day and counting the number of eggs that were laid for each animal.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.08.013.

Author Contributions

T.S.-V., A.d.L., S.I., and F.J.S. conceived the experiments. S.I. performed bioinformatics analyses. T.S.-V., A.d.L., M.S., and B.H. performed the *C. elegans* experiments. T.S.-V., A.d.L., S.I., and F.J.S. analyzed the data. T.S.-V., A.d.L., S.I., and F.J.S. wrote the manuscript.

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